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Review

Temperature-sensitive PSII and promiscuous PSI as a possible solution for sustainable photosynthetic hydrogen production ☆

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ABSTRACT

Sustainable hydrogen production in cyanobacteria becomes feasible as a result of our recent studies of the structure of photosystem I encoding operon in a marine phage. We demonstrated that the fused PsaJF subunit from the phage, substituted for the two separate subunits in *Synechocystis*, enabled the mutated PSI to accept electrons from additional electron donors such as respiratory cytochromes. In this way, a type of photorespiration was created in which the cell consumes organic material through respiratory processes and PSI serves as a terminal electron acceptor, substituting for cytochrome oxidase. We designed a hydrogen-producing bioreactor in which this type of photorespiration could utilize the organic material of the cell as an electron source for H_2 production. We propose, in parallel, to engineer cyanobacterial and/or algal strains with a temperature-sensitive PSII and enhanced respiration rates to achieve efficient and sustainable hydrogen production. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial.

1. Introduction

Oxygenic photosynthesis, the Earth's major producer of both oxygen and organic matter, is a principal player in the development and maintenance of life [1–3]. Producing oxygen and assimilating carbon dioxide into organic matter determines, to a large extent, the composition of our atmosphere and provides all life forms with essential food and fuel. During the onset of oxygenic photosynthesis approximately 3.5 billion years ago, our planet was under anaerobic conditions, and most of the elements were suspended in their reduced-valence states, which made them water-soluble. Thus, the atmosphere was rich in nitrogen in the form of ammonia (NH_3) or N_2, carbon as CO or CO_2, and oxygen as H_2O [4], and the surface was covered by water and minerals, including metal ions. Oxygenic photosynthesis used light energy and water to produce oxygen and hydrogen ($2H_2O \rightarrow 2H_2 + O_2$). Another 1.5 billion years passed before metal compounds were oxidized and free oxygen released into the atmosphere [5,6]. During this long period of time, oxygenic photosynthesis suffered a shortage of oxidized electron acceptors, such as metal ions, NO_3^-, and SO_4^{-2}, which are plentiful on the current Earth surface. Arguably, protons served as a sink for the excess electrons, and most of the hydrogen that was produced left Earth because it is light enough to gain the escape velocity of 11.2 km/s at normal temperature [7].

The question of whether we can utilize artificial photosynthetic hydrogen generation for the production of clean fuel has to find an answer to two major obstacles before even considering this option. One obstacle is the spatial separation of oxygen and hydrogen production that prevents combustion. The second obstacle is to obtain efficient light energy conversion that will make the process economically viable.

Upon the discovery of a marine phage that carries an operon encoding the PSI subunits [8], we set out to construct a phage-like PSI in *Synechocystis* in which the PsaF and PsaJ subunits are fused. In the transformed *Synechocystis*, the PsaJF fusion protein enabled the mutated PSI to accept electrons from additional sources, such as externally added respiratory cytochromes. In this way we can generate a form of photorespiration. This process is not to be confused with the photorespiration created by the reaction of Rubiso with oxygen. The mutated PSI allows the respiratory electron transport chain to transfer reducing equivalents to the photosynthetic electron transport chain. Since PSI replaces cytochrome C oxidase as a terminal electron acceptor no oxygen is consumed and this process can function without oxygen.

The wild-type (WT) and mutant *Synechocystis* PSI were purified, crystallized, and solved to low resolution, which showed the fused protein in the mutant strain and the separate two subunits in the WT strain (Mazor et al., in preparation). Moreover, several structural alterations in the mesophilic PSI were observed in the crystal structure of the WT compared to the solved structure of the thermophilic

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strain [9]. We propose that, in conjunction with engineering a cyanobacterium with a temperature-sensitive PSII and enhanced respiratory rates, efficient and sustainable hydrogen production can be achieved. Here, we propose a theoretical and practical design for cyanobacterial and microalgal bioreactors that may fulfill the above requirements.

2. General considerations

Sustainable energy should meet the needs of the present without compromising the ability of future generations to meet their needs. Therefore, by definition, plans for future energy production should take into account not only the next century, but also the next millennia. No single government can think in these terms; thus, it is the duty of the scientific community to strive for this goal. The guideline for this approach is plain and simple. Because the second law of thermodynamics governs life on earth, increasing entropy accompanies life. Future energy production should be generated in such a way that unavoidable garbage accumulation is dealt with. Most of the suggestions that are currently available do not fulfill this minimal demand, and therefore are polluting.

For the time being, sensible utilization of sunlight energy is the only available solution to the energy shortage problem. Approximately 120,000 TW (terawatts) of solar power reaches Earth's surface. Currently, global power consumption is roughly 15 TW, which is equivalent to the solar energy reaching 0.0125% of Earth's surface. Therefore, only 63,759 km^2 harvesting 100% of the average sun irradiation is sufficient for meeting all current energy demands. By building light-harvesting installations in arid areas not far from the equator, the increased light incidence will easily compensate for the 80% loss in the conversion of solar energy into utilizable energy. Numerous obstacles stand in the way of switching to solar energy as a feasible source, but in the future it should be a major part of global energy production. Biological energy production must be a major factor in harnessing solar energy.

However, a sensible approach has to be implemented without bending the second law of thermodynamics. Oxygenic photosynthesis, with its two photosystems (PSI and PSII), is a viable candidate for harnessing solar energy, and there are numerous theoretical and practical ways to do it. Two main attempts are now under extensive investigation; one is electricity production using isolated reaction centers or live cells, and the second is the production of biofuels (diesel, methane, butanol, ethanol, aviation fuel, and hydrogen). We think that the latter presents a more realistic approach and should be encouraged. However, if we fail to evaluate the promise of sustainable biofuel production through a realistic lens, exaggerated expectations will eventually undermine its legitimacy. A recent review signed by most of the leading photosynthesis scientists sets the limit of biomass production by oxygenic photosynthesis at 5% efficiency of the incident solar irradiance [10]. Producing biofuel from this material may reduce this efficiency to 1–2%. Among the biofuels, ethanol is already a commercial commodity, but biodiesel production by microalgae may be more realistic for the future [11]. U.S. oil consumption is roughly 1 billion tons per year. We estimate that at the realistic 1% efficiency, 3,600,000 ha or 36,000 km^2 will be required to fulfill this demand. If this approach is chosen, it is not out of reach, but we have to bear in mind that ethanol and biodiesel are not “blue” and their consumption will generate a net increase in CO_2. The only “blue” energy that is potentially available is photosynthetic hydrogen production.

3. Obstacles in bio-hydrogen production

All hydrogenic enzymes (hydrogenase and nitrogenase) make use of metallic centers, which are very strong reductants, and most of these enzymatic clusters are oxygen-sensitive. In particular, the faster [FeFe] hydrogenase is irreversibly inactivated by oxygen; therefore, the oxygenic activity of PSII is incompatible with biohydrogen production by this enzyme. Many biological mechanisms insulate oxygen-sensitive processes from atmospheric and cell-generated oxygen. These mechanisms include specialized cells or structures, such as heterocysts, in some of the multicellular nitrogen-fixing cyanobacteria [12]. In an extreme example, the marine diazotroph UCYN-A lost all of its PSII genes [13]. In another unicellular marine diazotroph, *Cyanothece sp. ATCC 5114*, nitrogenase-mediated hydrogen evolution can occur in the light, but the total amount of sustained H_2 photo-production is still unclear [14]. For the purpose of sustainable hydrogen production, a temporal separation between PSII-supported growth and H_2 production is desirable. One way to achieve this separation would be to select or design temperature-sensitive mutations in PSII. In such an organism, hydrogen production at the expense of the biomass that accumulated during photosynthetic growth will occur under anaerobic conditions. The selection of the organism that will be used for energy production is also critical; it should be either temperature or salt tolerant to prevent contamination by fast-growing organisms.

4. Cyanobacterial bioreactor for hydrogen production

Fig. 1 depicts a cyanobacterial bioreactor for hydrogen production. The four chambers enable physical separation between biomass accumulation, accompanied by oxygen evolution and biomass utilization for hydrogen production. At lower temperatures, cells will grow in a “regular” fashion, converting sunlight, water, and CO_2 into organic matter. Following this growth stage, cells will be shifted to a high temperature–oxygen free environment where oxygen evolution will shut down due to PSII inactivation and hydrogen will be generated. The elevated temperatures have the added benefit of limiting contamination and predation, which are serious problems in large (and small) scale bioreactors. In order to accommodate such drastic changes in the growth regime of the organism, we propose introducing several genetic changes which is detailed in Fig. 1 and described in greater detail below.

5. Possible genetic solutions to an H_2 production problem

The main challenge in adapting a cyanobacteria or algae species to our bioreactor is the anaerobic growth stage. Stopping PSII under certain circumstances is technically demanding, but it seems possible. However, without an active PSII complex, the vast majority of reducing equivalents for the photosynthetic electron transport chain are lost, which presents a serious problem. Perturbation in the photosynthetic electron transfer chain leads to severe growth phenotypes. The photosynthetic membranes of cyanobacteria and plants contain a special form of respiratory complex I called NDH-1 [15,16]. This complex lacks the NADH/NADPH oxidizing subunits and may function in a form of cyclic electron flow that is not yet fully understood. In theory, in order to compensate for the absence of PSII, an alternative quinone reductase, such as NDH-1, is needed. However, in practice, the levels of NDH-1 in the photosynthetic membranes are two orders of magnitude lower than that of PSI [16,17]. In addition, cyanobacterial cells contain a more complete respiratory electron transfer chain in their outer membrane; the rate of electron flow in these membranes is only about one-tenth the rate as that of the photosynthetic membranes [18]. None of these mechanisms can be used to fully compensate for the absence of PSII.

An alternative to complex I exists in many organisms. These proteins are monomeric NADH/quinone oxy-reductases lacking any proton pumping activity. *Synechocystis PCC6803* contains three such proteins, but their significance to electron transport is still not clear [19]. In the case of the baker yeast *Saccharomyces cerevisiea*, only these “alternative” proteins exist and the yeast cell can achieve very high rates of cellular growth by relying on their activity. In fact, one

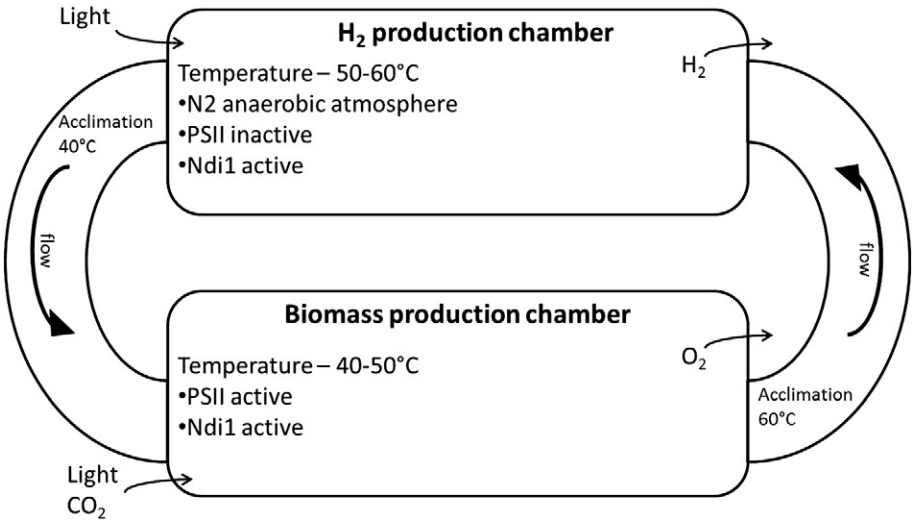

Fig. 1. A cyanobacterial hydrogen bioreactor. The reactor includes four chambers: two production chambers and two acclimation chambers. Cells will cycle between a high temperature for H_2 anaerobic production chamber and a lower temperature, aerobic growth chamber. The high activity of PSII in the lower temperature suppresses Ndi1 activity at lower temperatures.

of the yeast genes was previously used to rescue some complex I deficiencies in rat and human cell lines [20]. In very much the same way, we suggest replenishing the electrons lost from the photosynthetic electron transport chain as a result of the inactivation of PSII by using the heterologous expression of the alternative NADH/quinone oxido-reductase from yeast in cyanobacteria, which will supply the electrons to the photosynthetic quinone pool.

The utilization of organic matter to "feed" electrons into the PSI reaction center serves two purposes: to utilize the electrons generated from water splitting in hydrogen production and to alleviate the detrimental phenotypes resulting from knocking out PSII. Strictly speaking, a form of photorespiration is required in which the electrons coming from organic matter are energized by the absorbed light through PSI in order to support high rates of hydrogen production. H^+ reduction can either go through electron donation from soluble ferredoxin (Fd) or through a hydrogenase–Fd fusion which was recently found to enhance H_2 production rates significantly [21].

Simply using organic matter for H_2 production will probably result in a rather poor H_2 yield as the reduction potential of H^+/H_2 is low (−420 mV), about equal to that of reduced Fd and lower than that of $NAD(P)^+/NAD(P)H$ (−320 mV). Therefore, in order to produce large quantities of H_2 an excess of reactants is required. Though low pH values can be found in some cellular compartments, highly reducing molecules tend to react with many cellular components and, therefore, competition for reducing equivalents is a problem.

The terminal electron acceptor of PSI, F_B has a very low reduction potential (~−0.7 mV), and the potential difference between F_B and H_2 is more than sufficient to sustain high rates of H_2 production rate. Even if Fd is used as a mediator in electron transfer, a significant improvement over NADPH/NADH dependent reactions is expected.

Similar to mutations in PSII, one cannot simply redesign the PSI complex at will. Any mutation reducing the activity of PSI will hamper growth in any photosynthetic organism. Redirecting PSI activity towards hydrogen production requires a delicate balance between the activity dedicated to hydrogen production and other cellular processes. We think that many of the answers for achieving this balance lay in a recently discovered PSI complex encoded by a marine phage, which we think codes for a functional reaction center under the high-energy requirements conditions that follow phage infection of cyanobacteria.

The phage PSI operon contains eight PSI genes: PsaA-E and a unique PsaJF fusion subunit. All of the phage-encoded genes contain a large number of mutations relative to the known PSI sequences and are of great scientific interest. The most noticeable difference is the psaJF fusion protein, in which the N-terminus of PsaF is truncated [8]. These two subunits remained separated throughout the evolution of the PSI complex in both plants and cyanobacteria. The PsaF subunit of plant PSI contains a positive N-terminal loop that is absent from the cyanobacteria complex. This loop was shown to promote the fast phase of the electron transfer reaction between PSI and its electron donors in plants [22]. However, the role of the entire N-terminal domain of PsaF is not as well defined. This domain, together with a PsaA loop (624–633 in the Thermosynechococcus elongatus BP-1 [9] structure), are the two most prominent features of the rather flat luminal side of PSI. These two structural features have been proposed to coordinate the interaction between Cyt553/PC and P700 during electron transfer, and both are missing from the phage PSI complex [8]. However this coordination role appears to be rather minor as a PsaF deletion strain of *Synechocystis sp. PCC 6803* is capable of photoautotrophic growth, and a phage mimetic PsaJF fusion in *Synechocystis* does not confer any growth phenotype ([23] and our unpublished observation). Many marine phages code for an alternative form of the D1 protein from PSII [24]. D1 is probably the most susceptible point of the photosynthetic electron transfer chain, and so, PSII activity may limit phage replication in certain scenarios. The additional, phage coded, D1 genes enhance photosynthesis during phage infection [25]. We think that the phage PSI serves a similar purpose, to allow cells to maintain their photosynthetic activity when the PSII complex is compromised. This rescue can be achieved if the phage-encoded PSI can accept electrons from electron donors involved in respiration. The respiratory cytochrome C is typically a very basic protein, but it can be used as an electron donor for PSI in vitro and the rates of electron transfer are typically quite low compared to the native PSI donors.

Indeed we demonstrated that in the PsaJF *Synechocystis* mutant the rate of oxidation of externally added horse heart cytochrome C is markedly increased (2 to 4 times over the wild type complex) (unpublished results).

Removing the PsaF N-terminal domain and the luminal loop of PsaA should allow PSI to accept electrons from these very basic proteins. In this form of photorespiration, PSI is used to drive an electron transport chain dedicated to ATP production in which NDH-1, together with the b_6f (or bc_1) complexes, generate a proton gradient used solely for ATP production. This hypothetical electron transfer chain does not produce (or consume) O_2.

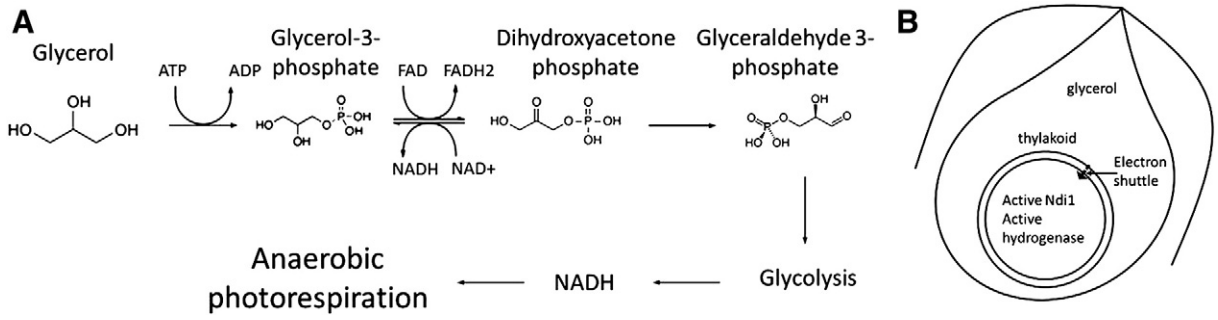

Fig. 2. Photorespiration change to photo-hydrogen production: A. A proposed scheme to convert glycerol into H_2. B. Schematic representation of the microalga cells utilizing electron shuttle from the cytoplasm into the stroma, the proposed shuttle can be built upon the preexisting malate–oxaloacetate shuttle.

6. An algae-based bioreactor

Similar principals can also be proposed for a microalgae-based reactor for hydrogen production. The main difference from the cyanobacterial reactor is the site of biomass accumulation. In cyanobacteria the biomass accumulates in a compartment accessible to the electron transport chain, whereas in algae the biomass equivalents have to cross two membranes to reach it. This can be achieved using one of the red-ox shuttles which already exist in the cell, such as the malate shuttle. In chloroplasts, the malate/oxaloacetate redox pair is used as an electron valve to transport reducing power outside of the chloroplast. However, as chloroplast can take up malate this redox shuttle can run in reverse if a sufficiently large electron sink exist within the chloroplast. A combination of a heterologously expressed active di1 protein and an active hydrogenase can serve as such an electron sink (Fig. 2B). An Ndi1/hydrogenase combination can reverse the flow of reducing equivalents into the chloroplast using malate while in permissive temperature the activity of Ndi1 will be minor because PSII will reduce the quinone pool efficiently.

Currently, the organism of choice for most large-scale growth enterprises is algae rather than cyanobacteria. Particularly attractive organisms for biotechnological applications are members of the *Dunaliella* genus [26]. Members of this genus can thrive under extremely "salty" or acidic conditions at relatively high temperatures. These extreme growth requirements can be used to protect the culture from contamination and predation. Certain *Dunaliella* species provide a commercial source for β-carotene, a chemical that they accumulate in very large amounts under certain growth conditions [26]. The ability of *Dunaliella* to sustain growth at high salinity is attributable to its ability to increase its internal glycerol concentration in order to sustain an osmotic balance with its environment, as well as several other metabolic adaptations [27–30]. The internal glycerol concentration in *Dunaliella* can reach 7–8 M under high salt growth conditions, however it can tolerate significant glycerol concentration drops [29]. This provides an opportunity to drive useful reactions using this large pool of glycerol by exogenously introducing enzymes to divert it towards hydrogen production. In Fig. 2, we describe a synthetic pathway based on the existing routes for glycerol utilization in cells to divert electrons from glycerol back to the photosynthetic electron transfer chain, where they can be used to drive H_2 production.

The above genetic manipulations, the cyanobacterial and algal strains, and the proposed bioreactors are compatible with the principle requirements for obtaining efficient and sustainable photosynthetic bio-hydrogen production. Time will tell whether this approach can be translated into economically viable, large-scale energy source.

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